CLINICAL SCIENCE

DNA SEQUENCING CONFIRMS THE INVOLVEMENT OF *LEISHMANIA* (*L.*) *AMAZONENSIS* IN AMERICAN TEGUMENTARY LEISHMANIASIS IN THE STATE OF SÃO PAULO, BRAZIL

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Medeiros AR, Silva Jr WA, Roselino AM. DNA sequencing confirms the involvement of *Leishmania (L.) amazonensis* in american tegumentary leishmaniasis in the state of São Paulo, Brazil. Clinics. 2008;63:451-6.

INTRODUCTION: American tegumentary leishmaniasis (ATL) represents one of the most important public health issues in the world. An increased number of autochthonous cases of ATL in the Northeastern region of São Paulo State has been documented in the last few years, leading to a desire to determine the *Leishmania* species implicated.
METHODS: PCR followed by DNA sequencing was carried out to identify a 120bp fragment from the universal kDNA minicircle of the genus *Leishmania* in 61 skin or mucosal biopsies from patients with ATL.
RESULTS: DNA sequencing permitted the identification of a particular 15bp fragment (5' …GTC TTT GGG GCA AGT... 3') in all samples. Analysis by the neighbor-joining method showed the occurrence of two distinct groups related to the genus *Viannia* (*V*) and *Leishmania* (*L*), each with two subgroups. Autochthonous cases with identity to a special *Leishmania* sequence not referenced in Genbank predominated in subgroup *V*.1, suggesting the possible existence of a subtype or mutation of *Leishmania Viannia* in this region. In the subgroup *L*.2, which showed identity with a known sequence of *L. (L.) amazonensis*, there was a balanced distribution of autochthonous and non-autochthonous cases, including the mucosal and mucocutaneus forms in four patients. The last observation may direct us to new concepts, since the mucosal compromising has commonly been attributed to *L. (V.) braziliensis,* even though *L. (L.) amazonensis* is more frequent in the Amazonian region.
CONCLUSIONS: These results confirm the pattern of distribution and possible mutations of these species, as well as the change in the clinical form presentation of ATL in the São Paulo State.

KEYWORDS: Tegumentary. Leishmaniasis. Phylogenetic analysis. *L. (L.) Amazonensis*, *L. (V.) Braziliensis*. Molecular epidemiology.

INTRODUCTION

Leishmaniasis persists as a concern of public health. American tegumentary leishmaniasis (ATL) has been listed among the six most prevalent infectious-parasitic diseases in the world, with a growing number of cases in Brazil.[1]

It is characterized by cutaneous, mucosal, and mucocutaneous forms, caused by the subgenus *Viannia* (*V*) and *Leishmania* (*L*). *L. (V.) braziliensis* predominates in a wider geographical distribution than other species in the subgenus *Viannia* in Brazil. In the subgenus *Leishmania*, *L. (L.) amazonensis* is primarily observed in the Amazonian forest region. Its distribution has recently increased, with autochthonous cases described in the southeastern region, where the human disease is relatively rare and presents in the localized and diffuse cutaneous forms.[2,3]

Molecular biology data have demonstrated the complexity of the population structure of the *Leishmania* species and their vectors. Studies have demonstrated the occurrence of genetic polymorphisms among strains of *L. (V.) braziliensis* and *L. (L.) amazonensis*.[4-6] Dujardin et al. (2002) suggested that leishmania rearrange and adequately

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adjust their genome structure for the purpose of fitness and pathogenicity.[7]

Since an increased number of ATL autochthonous cases has been diagnosed in the Northeastern region of the State of São Paulo (SP), it has become important to identify the *Leishmania* species involved as well as its pathogenecity.[1,8] For this proposal, PCR has been utilized with great specificity and sensibility.[9-12]

MATERIALS AND METHODS

Sixty-one patients -- each with a clinical epidemiological and laboratory diagnosis of ATL attended at the Dermatology outpatient clinic at the Hospital of Clinics, Faculty of Medicine of Ribeirão Preto, University of São Paulo, between January 1993 and June 2002 -- were included in the study after giving informed consent to participate. All patients and procedures were approved by the Ethics Committee (HCRP n.4134/02).

Skin and/or mucosal biopsy

Skin or mucosal biopsies were obtained from the border of the ulcerated lesion using a 4 mm punch under aseptic conditions and with local anesthesia. The specimen was frozen at -70ºC for later PCR processing.

Sample preparation for PCR

A 2 mm fragment of the frozen biopsy was placed in 1 mL of proteinase K (PK) buffer (0.1 M Tris, pH 8.0, 0.1 M EDTA, 1% SDS, and PK at a final concentration of 20 mg/mL). The samples were incubated at 55ºC overnight and then heated to 95ºC for 10 min for PK inactivation. Phenol and chloroform extraction and ethanol precipitation were then performed. The precipitated DNA was then resuspended in 30 µL of distilled water.

Polymerase chain reaction (PCR)

The primers 5'- (G/C) (G/C) (C/G) CC (A/C) CTA T(A/T) TTA CAC CCA ACC CC – 3' and 5'- GGG GAG GGG CGT TCT GCG AA – 3' (Ultrachem) were used for PCR. These primers anneal the conserved region of the kDNA minicircle and are specific for *Leishmania spp*.. PCR was performed in a final volume of 30 µL containing 750 mM Tris-HCl, pH 9.0, 15 mM $MgCl_2$, 200 mM $(NH_4)_2SO_4$, 1% Tween 20.5 mM dNTP, 25 pmoles of each primer, and 1 U Taq DNA polymerase. We then added 5 µL of the DNA sample to this mixture. Amplification was performed in a model 9700 thermocycler (Applied Biosystems). An initial denaturation of 3 min and 3 s at 94ºC was followed by thirty-five cycles: 94ºC for 30 s, 60ºC for 1 min, 72ºC for 1 min, and a final 10 min extension, ending at 4ºC. Each reaction included a control without DNA and a known positive control. The positive control was performed with a lysate sample of *Leishmania* culture: *L. major* (LV 39 clone 5-RHO/54/59/P) and *L. (L.) amazonensis* (IFLA/BR/67/PH8).

The PCR products were separated by electrophoresis in 1.5% agarose gel with 1X TBE (Tris-borate – ethylenediaminetetraacetic acid) buffer and stained with ethidium bromide.

DNA Sequencing

The PCR products were sequenced using the sense primer and the DNA Sequencing Kit Big Dye Terminator™ (Applied Biosystems) according to the manufacturer's instructions. Sequencing was performed with an ABI Prism 310™ Genetic Analyzer (Applied Biosystems).

Sequence editing and alignment

The sequences were edited and aligned using the BioEdit program, v5.0.9 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).[13]

Phylogenetic analysis

The similarities among the *Leishmania* sequences of the 61 samples were determined by the neighbor-joining (NJ) method.[14] This method uses a distance matrix between sequence pairs to generate dendograms. In this analysis, we included *Leishmania* sequences obtained from cultures (*L. major* and *L. (L.) amazonensis*) as well as Genbank sequences used as reference for different *Leishmanias*: *L. guyanensis* (M87316), *L. panamensis* (M87314), *L. braziliensis* (M87315), *L. peruviana* (M87317), *L. amazonensis (*M21326), *L. major* (J04654), *L. donovani* (AJ010075), and *L. lainsoni* (AF088234). For this analysis, we used the P-distance matrix, which is based on the difference between sequences when compared pair-wise, employing the Molecular Evolutionary Genetic Analysis (MEGA) system described by Kumar et al. (2001).[15]

RESULTS

Sequencing permitted the identification of a particular 15 bp sequence (5'...GTC TTT GGG GCA AGT...3') in all samples. Phylogenetics analysis using the NJ method with a bootstrap of 1000 replicates permitted us to obtain

a consensus dendogram in which two groups were defined, denoted by *V* and *L* (Figure 1). Group *V* presents two subgroups, *V*.1 and *V*.2, which are characterized by the presence of the samples numbered (n) 60, 49, 51, 25,

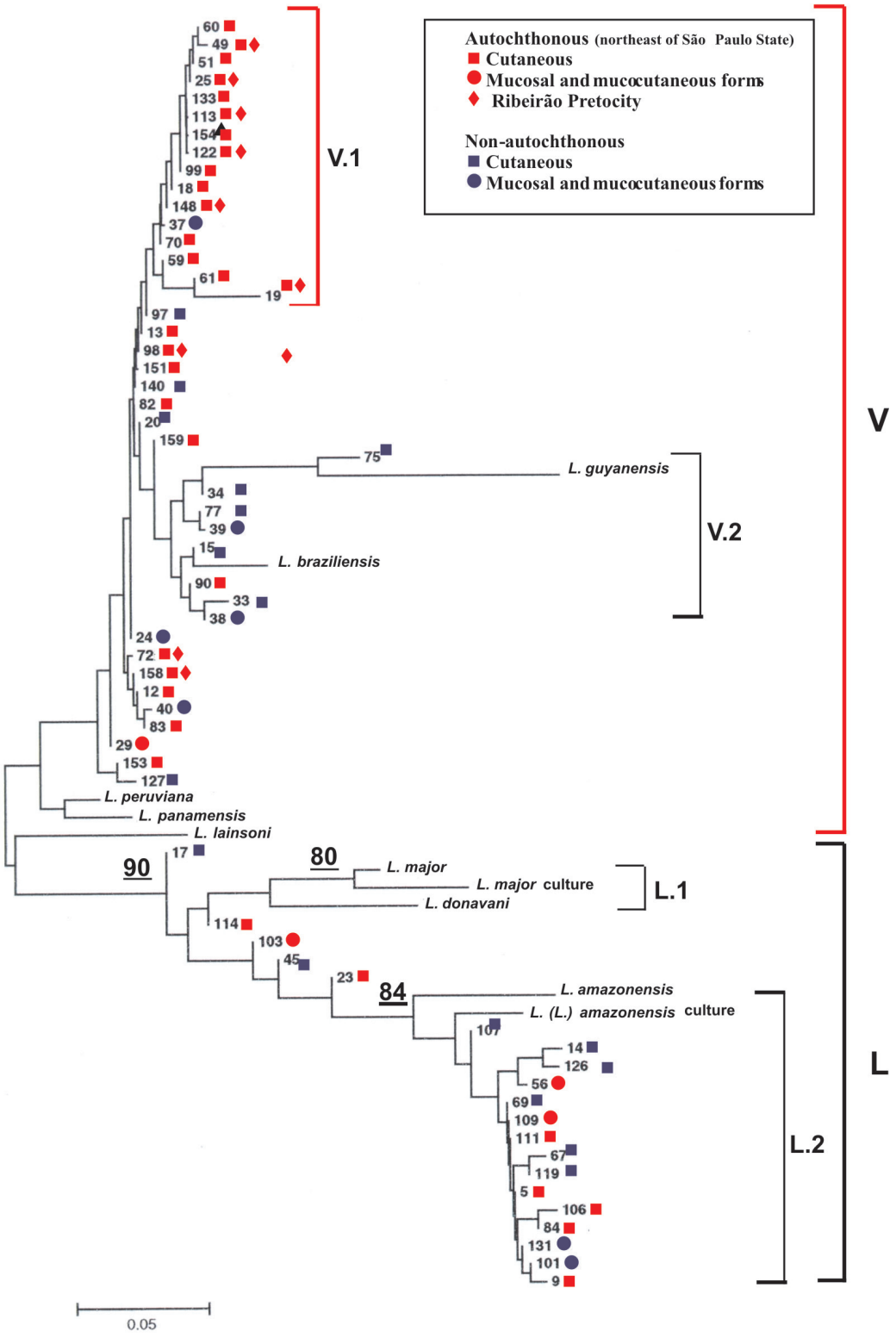

Figure 1 - Consensus dendogram obtained by neighbor-joining analysis with a bootstrap of 1000 replicates (bootstrap values greater than 80% are shown), characterizing the association of groups and subgroups of *Leishmania* species in terms of origin and clinical form

133, 113, 154, 122, 99, 18, 148, 37, 70, 59, 61, 19, and the samples 75, 34, 77, 39, 15, 90, 33, 38, respectively, together with the reference sequences of *L. guyanensis* and *L. braziliensis* in the latter. The same was observed in group *L*, with the definition of two subgroups: *L*.1, characterized by the reference sequences of *L. major*, *L. donovani*, and the *L. major* culture, with *L*.2 containing the samples 107, 14, 126, 56, 69, 109, 111, 67, 119, 5, 106, 84, 131, 101, and 9, plus the reference sequence and the *L. (L.) amazonensis* culture.

The distribution of subgroup *V*.1, which contained 16 samples, showed a predominance of autochthonous cases and of cutaneous form. Six of these were from the municipality of Ribeirão Preto, and only the sample n.37, a mucocutaneous form, originated from the State of Minas Gerais. Among the eight samples composing subgroup *V*.2, only n.90 was autochthonous. One mucosal form (n.39) and one mucocutaneous form (n.38) were present in addition to the reference sequences of *L. braziliensis* and *L. guyanensis*. Of the 15 samples that composed subgroup *L*.2, with the reference sequence and the culture of *L. (L.) amazonensis*, seven corresponded to autochthonous cases (five with cutaneous, one with mucosal, and one with mucocutaneous forms). In the remaining cases, there was one patient with a mucocutaneous form of unknown origin and one patient with a mucosal form from the State of Pará.

There were three samples of disseminated forms: n.34, a patient with a cutaneous form from Minas Gerais, which presented identity with subgroup *V*.2; n.17, a patient with a cutaneous form from Goiás co-infected with HIV; and n.103, a patient with a mucocutaneous form from the northeast region of the State of São Paulo. The latter two samples were assigned to the group *L*, having no correlation with a subgroup or identity with a reference sequence. It is important to note that in addition to n.17, n.38, a patient with mucocutaneous form from the State of Bahia, presented co-infection with HIV and was assigned to the subgroup *V*.2.

DISCUSSION

Since 1913, when *Leishmania (V.) braziliensis* was identified as the responsible parasite for epidemic ulcers in Bauru (a city in SP), that particular species has been implicated as the etiologic agent of ATL in SP.[2,16-20] Yoshida et al. (1979)[21] were the first to isolate leishmania from a wild animal, the *Didelphis marsupialis aurita* (identified as belonging to the subgenus *Leishmania*). In 1988, Tolezano et al.[18] isolated leishmania identified as *Leishmania amazonensis* from *Akodon sp*. (Rodentia, Cricetidae) captured in the Ribeira Valley region of SP. In agreement with Cupolillo et al.,[22,23] the dendogram obtained by the NJ method consisted of two groups in which the distribution of the reference *Leishmania* was identified by subgenus *Viannia* (*V*) and *Leishmania* (*L*). In subgroup *V.1*, consisting exclusively of patients' sample sequences, 15 (93.75%) were autochthonous cases with the cutaneous form. It should be pointed out that six (40%) of the patients lived in Ribeirão Preto, SP. Surprisingly, the data in subgroup *L.2* were obtained from 15 patients' sample sequences in addition to the reference sequence of *L. amazonensis*. Of these, seven (46.76%) corresponded to autochthonous cases.

Regarding the clinical forms, the cutaneous form showed uniform distribution amongst the groups. The mucosal and mucocutaneous forms also showed this distribution pattern. Four cases with the mucosal and mucocutaneous forms in subgroup *L.2* were observed, which maintained identity with *L. amazonensis*. Of these, two patients represented autochthonous cases from the northeastern region of SP.

In Brazil, mucosal and mucocutaneous forms of leishmaniasis can be manifested exclusively as mucosal compromising or as a concomitant cutaneous lesion that may be concurrent or contiguous with the mucosal region, respectively, which are attributed to *L. (V.) braziliensis*. Nevertheless, *L. (L.) amazonensis* and *L. (V.) guyanensis* species can also be involved in mucosal form.[2] Of the four mucosal forms observed in subgroup *L.2*, only two showed a concomitant cutaneous lesion, with the possibility of mucosal compromise resulting from contiguity. The mucosal lesion was isolated in the other two cases, an observation that disagrees with past literature.

In our study, there were three cases with a disseminated form, one of them located in subgroup *V.2,* which maintains identity with the references *L. braziliensis* and *L. guyanensis*. The other cases, one cutaneous and the other a mucocutaneous form, were located in the group that identified with the subgenus *Leishmania*.

The autochthonous cases in subgroup *L*.2, which maintains identity with the *L. amazonensis* complex, showed an unexpected result, since this complex has been predominantly identified in the Amazon region. It is tempting to propose that in subgroup *V*.1, which contains strains from autochthonous cases (especially in the municipality of Ribeirão Preto) and does not identify with a particular *Leishmania Viannia* species, there may be a subtype or a mutation of the subgenus *Viannia* in the region under study. More extensive studies are needed in order to isolate and identify the *Leishmania* involved and to correlate it with wild vectors and hosts.

The primers used in the present study amplify a 120bp fragment in all *Leishmania* species.[24] It is important to note

that the sense primer is complementary to the universal sequence of the kDNA minicircle. The initial intention was to sequence more common fragments of the kDNA minicircle in a search for mutations. These mutations were observed in several samples, perhaps explaining the diversity of the strains in the listed subgroups.

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